Quantitative Micro Determination of Alcohols as Esters of Pyruvic Acid 2,6-Dinitrophenylhydrazone

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Methods for the quantitative colorimetric determination of micro amounts of alcohols have been limited to only a few reagents (1–4). Although in itself the quantitative determination of micro amounts of alcohols (colorimetrically or otherwise) is of considerable utility, it tells nothing of the types of classes or of the individual alcohols present. To date there has been no quantitative method which combines this desirable feature. A recent series of reports from this laboratory centered around pyruvic acid chloride 2,6-dinitrophenylhydrazone,[1] a new acylating and potentially valuable reagent for isolation and identification studies. Preparation of derivatives of primary, secondary, and tertiary alcohols (5), primary and secondary amines (6), and thiols (7) in good yield on a semimacro scale was described. It was subsequently possible to effect separation of the 3 major classes of alcohols from each other (8) and also from the amine and thiol derivatives (9). In addition, homologous series of all classes of the derivatives were separable by thin-layer partition (10) and gas-liquid (11) chromatography.

Besides lending themselves admirably to fractionation, the derivatives were highly colored and possessed a constant molar absorptivity within a class, and, in the case of alcohols, between classes. In view of these desirable features, it seemed worthwhile to investigate the quantitative potentialities of derivative formation as a colorimetric method for the determination of alcohols at the micromole level.

REAGENTS

Benzene, thiophene-free, ACS grade, was obtained from J. T. Baker Co.,[2] Phillipsburg, N. J.; aluminum oxide, acidic, Brockman activity

[1] Pyruvic acid chloride 2,6-dinitrophenylhydrazone in this paper will also be referred to as (the) reagent or (the) acid chloride.

[2] Mention of brand or firm names does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

grade I (Baker), was partially deactivated with 8% distilled water and equilibrated at least 16 hr before use; pyruvic acid chloride 2,6-dinitrophenylhydrazone was prepared in this laboratory (8) (this reagent is now commercially available from J. T. Baker Chemical Co.); triethylenediamine (1,4-diazabicyclo(2.2.2)octane) was purchased from Matheson, Coleman & Bell, East Rutherford, N. J.; calcium hydride was a product of Fisher Scientific Co., Silver Spring, Md. The alcohols used in this study were purchased from various sources (see Table 1) and were checked for purity by gas-liquid chromatography. All liquid alcohols listed in Table 1 were at least 95% pure by this technique, and the majority were better than 97% pure. Cholesterol was purified via the dibromide (12); lanosterol was contaminated with about 40% dihydrolanosterol; dihydrolanosterol was prepared from commercial lanosterol by reduction with hydrogen in ethanol using a palladium catalyst (13) and was pure by gas-liquid chromatography after recrystallization from acetonitrile; *cis*-9,10-epoxy-1-octadecanol was a gift from Dr. L. S. Silbert, EURDD, ARS, USDA, Philadelphia, Pa.; the methyl hydroxystearates were kindly donated by Dr. A. P. Tulloch, National Research Council of Canada, Prairie Regional Laboratory, Saskatoon, Saskatchewan, Canada.

EXPERIMENTAL

Preparation of alcohol-free benzene. Benzene is rendered completely free of alcohols (and also thiols and amines, if present) as follows: 2 gm of chromic anhydride (Fisher) is dissolved in 24 ml of distilled water in an 8 in. mortar. Then 30 gm of analytical-grade Celite (Johns-Manville Co., Baltimore, Md.) is added and the mixture ground for

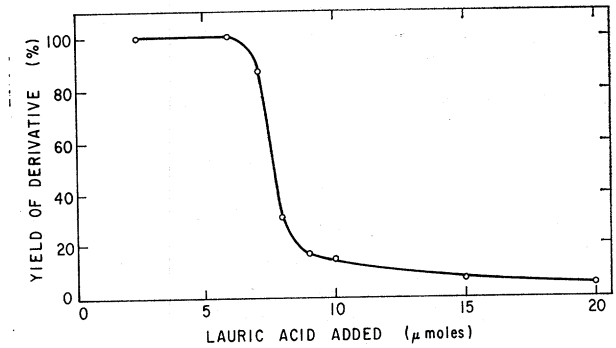

FIG. 1. Effect of lauric acid on reaction of pyruvic acid chloride 2,6-dinitrophenylhydrazone with 2,5-dimethyl-3-hexanol. Concentration of acid chloride = 9.4 μmoles; concentration of 2,5-dimethyl-3-hexanol = 2 μmoles; concentration of triethylenediamine = 7.2 μmoles; total volume = 1.8 ml.

several minutes. The Celite is scraped from the sides and bottom of the mortar and reground for a few minutes. This process is repeated until the Celite is homogenously yellow. The impregnated Celite is added in small portions to a glass column (approximately 34 × 2.5 cm) containing a coarse fritted-glass disc or a plug of glass wool. The Celite is tamped tightly between additions. The flow rate should be approximately 20–25 ml/hr. The column will turn chocolate brown from top to bottom as benzene is purified. As long as a yellow portion remains, more benzene can be purified. A chromic acid column prepared as described can purify about 8 gal of the benzene specified. The solvent is further purified by passing the effluent from the chromic acid column over a column of basic alumina (Woelm, activity grade I) using 25 gm of alumina per liter of benzene. The effluent from this column, after discarding the first 100 ml, is distilled in an all-glass apparatus. This distillate is stored over CaH_2.

Preparation of reagents. A benzene solution of pyruvic acid chloride 2,6-dinitrophenylhydrazone is prepared to contain 23.5 μmoles/ml. The solution is stored over a few pellets of CaH_2. A benzene solution of triethylenediamine is made up to contain 18.5 μmoles/ml and is also stored over a few pellets of CaH_2.

Preparation of alcohol solutions. Solutions of the alcohols were prepared in purified benzene to contain up to 5 μmoles/ml.

General assay procedure. Assays were conducted in 10 ml test tubes fitted with Teflon-lined screw caps. Using a single 0.5 ml graduated pipet, 0.00, 0.25, 0.50, 0.75, and 1.00 ml aliquots of the alcohol solution were pipetted into the tubes. This was followed by 0.4 ml (9.4 μmoles) of the acid chloride solution, permitting the solution to run down the side while rotating the tube. Benzene was added in the same manner to the appropriate tube to bring the volume to 1.4 ml. A small pellet or two of CaH_2 was added, the tubes stoppered and let stand until gas evolution ceased. When dry, 0.4 ml (7.2 μmoles) of the triethylenediamine solution was added while carefully shaking the tube. This solution turns bright red on addition of the base, becomes turbid, and eventually fades to a lighter color.

Isolation and quantitation of derivatives. The tubes can be analyzed immediately following addition of the base or at any reasonable time afterward. It is recommended, however, that the tubes be analyzed the same day, since the blank appears to be slightly higher when the reaction mixture is held overnight. The contents of the tube are transferred to a chromatographic column (approximately 1 cm i.d. × 17 cm) containing about 2.5 gm of alumina which had been poured dry into the column. The effluent is collected in a 10 ml glass-stoppered volumetric flask.

All color below the reagent band, (which remains at the top of the column) and below a slow-moving impurity band (see "Discussion") is collected. The solution is made to the mark and read against a blank at 400 mμ. Concentration of derivative is calculated using $E = 5926$ for primary and secondary alcohol derivatives and $E = 5890$ for tertiary alcohol derivatives (1).

For analyses conducted on less than 0.5 μmole of alcohol, the effluent from the alumina column was collected in small beakers, the solvent evaporated under a stream of N_2, and the residue read in a convenient volume, usually 3 ml, against another blank treated in the same manner.

Assay of large volumes of dilute alcohol solutions. To test the efficiency of the method on a larger volume of a dilute solution of the alcohols, the following procedure was adopted: 23 ml of benzene, 0.4 ml of the acid chloride solution, and the alcohol solution were dried overnight or longer over a few pellets of CaH_2 in a 25 ml glass-stoppered volumetric flask. It is important that no water be present (i.e., no gas bubbles be evident), otherwise less than quantitative yields may result. The base (0.4 ml) was then added, and the solution mixed and analyzed as above, except that the column dimensions were altered: 10 gm of alumina contained in a column (1.6 cm i.d. $\times$ 12 cm) was utilized. The effluent was collected in a beaker and evaporated to below 10 ml. The solution was then transferred to a 10 ml volumetric flask and made to the mark for spectrophotometric evaluation.

Purity of derivatives. Each alcohol derivative prepared in the assay procedure was checked by thin-layer chromatography. Both the alkaline and neutral partition systems described by Schwartz *et al.* (14) were utilized. The plates were developed in the solvent system, hexane/benzene (65:35) saturated with polyethylene glycol 400. In most cases authentic crystalline derivatives were run on adjacent spots.

RESULTS AND DISCUSSION

Table 1 lists 46 alcohols representing a variety of classes which were assayed in 1.8 ml of benzene. Also in Table 1 is listed the range over which an alcohol has been investigated, the number of determinations performed within the range, and the yield. The latter is the average of the yields obtained at the various concentrations assayed within the range. When the amount of alcohol assayed was plotted against the yield of derivative, a linear relationship was found for all of the alcohols in Table 1.

From a practical standpoint, determination of micro amounts of alcohols in a relatively large volume of solution would be not only desirable but in most instances essential. In Table 2 are listed a number

TABLE 1

Reaction of Various Alcohols with Pyruvic Acid Chloride 2,6-Dinitrophenylhydrazone in 1.8 ml of Benzene

Compound	Source	Range investigated, μmoles	No. of assays within range	Av. yield, %	Av. dev., %
Isobutyl alcohol	Matheson[a]	0.18–3.60	8	98.5	±0.6
3β-Cholestanol	"	0.95–3.80	4	100.5	±0.7
Cholesterol	Aldrich[b]	0.34–3.40	5	104.0	±0.2
Cinnamyl alcohol	Matheson	0.20–4.00	9	93.2	±1.0
Citronellol	Aldrich	0.15–3.10	8	96.0	±0.7
Cyclohexanol	Matheson	0.18–3.60	8	94.5	±0.8
1-Decanol	Hormel[c]	0.20–4.00	5	96.6	±1.0
3-Decanol	Aldrich	0.20–4.00	8	103.0	±0.2
DL-1,2-*o*-Dihexadecyl glycerol	Fluka[d]	0.10–4.00	8	100.0	±0.5
Dihydrolanosterol	See text	1.00–4.00	4	98.0	±0.6
2,2-Dimethyl-3-hexanol	Aldrich	0.90–3.60	4	94.5	±0.2
2,2-Dimethyl-3-octanol	"	0.30–4.00	7	100.0	±0.6
2,2-Dimethyl-1-propanol	"	1.00–4.00	4	93.3	±0.8
1,3-Dipalmitin	Supelco[e]	0.56–2.82	4	95.0	±1.3
cis-9,10-Epoxystearic alcohol	EURDD[f]	0.18–3.6	4	97.8	±0.7
3-Ethyl-3-heptanol	Aldrich	0.35–3.5	5	96.0	±0.6
Farnesol	Mann[g]	0.19–3.80	8	97.6	±1.9
2-Heptanol	Aldrich	0.18–3.60	6	97.4	±0.3
4-Heptanol	"	0.36–3.60	5	94.7	±1.2
L-Isopulegol	Aldrich	0.17–3.39	8	95.7	±0.6
Lanosterol	"	1.00–4.00	4	98.5	±0.5
L-Menthol	"	0.20–4.00	8	102.0	±0.8
3-Methyl-5-hexen-3-ol	"	0.36–3.60	5	96.6	±0.9
Methyl 6-hydroxystearate	See text	1.05–4.20	4	103.0	±1.2
Methyl 7-hydroxystearate	"	0.39–1.95	5	97.3	±0.8
Methyl 17(+)-hydroxystearate	"	0.30–4.20	5	99.4	±0.5
Methyl 12-hydroxystearate	"	0.50–5.00	5	100.0	±0.5
3-Methyl-2-hexanol	"	0.18–3.60	9	100.0	±1.1
2-Methyl-2-nonanol	Chemical samples[h]	0.87–3.48	4	95.0	±1.1
3-Methyl-4-nonanol	Aldrich	0.83–3.34	4	99.0	±0.75
2-Methyl-3-octanol	"	0.33–3.30	8	99.2	±0.8
2-Methyl-3-pentanol	"	0.21–4.30	8	96.0	±0.5
4-Methyl-2-pentanol	Aldrich	0.10–4.00	9	98.2	±0.9
3-Methyl-1-pentanol	"	0.97–3.88	4	96.3	±1.8
2-Methyl-1-penten-3-ol	Chemical samples	0.32–3.20	5	94.5	±0.5
4-Methyl-4-penten-2-ol	"	0.36–3.60	5	96.6	±1.3
4-Methyl-1-penten-3-ol	Aldrich	0.44–4.40	5	96.0	±0.7
2-Methyl-4-penten-2-ol	"	0.40–4.00	5	92.8	±0.3
2-Nonanol	"	0.28–2.80	5	92.4	±1.4
5-Nonanol	"	1.00–4.00	8	95.4	±0.5

TABLE 1 (*Continued*)

Compound	Source	Range investigated, μmoles	No. of assays within range	Av. yield, %	Av. dev., %
2-Nonadecanol	Lachat[i]	0.39–3.90	5	96.1	±1.3
Oleyl alcohol	Hormel	0.19–3.76	8	103.0	±1.1
β-Phenylethanol	Matheson	0.15–3.00	7	97.7	±1.5
3-Phenyl-1-propanol	"	1.00–4.00	4	98.0	±1.0
2-Propanol	Baker[j]	0.15–3.78	7	99.7	±0.6
2-Undecanol	Aldrich	0.33–4.40	8	101.5	±0.8

[a] Matheson, Coleman & Bell, East Rutherford, New Jersey.
[b] Aldrich Chemical Co., Milwaukee, Wisconsin.
[c] Hormel Institute, Austin, Minnesota.
[d] Fluka A. G., Buchs, Switzerland.
[e] Supelco, Inc., Bellefonte, Pennsylvania.
[f] Eastern Utilization Research and Development Division, U. S. Dept. of Agriculture, Philadelphia, Pennsylvania.
[g] Mann Research Laboratories, New York, New York.
[h] Chemical Samples, Inc., Columbus, Ohio.
[i] Lachat Chemical Co., Chicago, Illinois.
[j] J. T. Baker Co., Phillipsburg, New Jersey.

of alcohols assayed in 25 ml of solution as described earlier. Quantitative acylation takes place under these conditions, which is advantageous, since it can eliminate the need, in certain cases, of evaporation or other manipulation to concentrate the alcohols.

Specificity. As Table 1 clearly shows, the acid chloride reagent does not discriminate between alcohols, acylating tertiary alcohols just as readily as it does primary and secondary alcohols. This fact alone should make pyruvic acid chloride 2,6-dinitrophenylhydrazone particularly valuable for estimating hydroxyl groups. 3,5-Dinitrobenzoyl chloride, which is commonly used in this regard (1,15), has the serious disadvantage of reacting very slowly with tertiary alcohols.

Of the classes tried which are not included in Table 1, only α-hydroxy acid methyl esters (both aliphatic and aromatic) and phenols (phenol, α- and β-tocopherols) were found not to react quantitatively with the reagent. Yields were in the vicinity of 20–40% and deviations from linearity were noted when concentration was plotted against yield of derivative.

The highly hindered tertiary alcohol, triphenylmethanol, did not react at all under the stated conditions.

Reaction conditions. Acylation of alcohols by the reagent is apparently instantaneous upon addition of triethylenediamine to the reaction vessel

TABLE 2
Reaction of Various Alcohols with Pyruvic Acid Chloride 2,6-Dinitrophenylhydrazone in 25 ml of Benzene

Compound	Amt. investigated, μmoles	Yield, %
3β-Cholestanol	1.12	103
Cholesterol	0.85	103
Cinnamyl alcohol	1.00	87
Citronellol	0.46	93
Cyclohexanol	2.00	98
3-Decanol	1.00	104
2,2-Dimethyl-3-hexanol	0.89	94
2,2-Dimethyl-1-propanol	1.00	99
3-Ethyl-5-hexen-3-ol	1.00	97
3-Ethyl-3-pentanol	0.93	97
Furfuryl alcohol	0.91	90
Isobutyl alcohol	0.92	101
L-Isopulegol	0.84	99
Lanosterol	1.00	96
3-Methyl-3-heptanol	0.92	100
Methyl 6-hydroxystearate	1.88	100
Methyl 12-hydroxystearate	1.28	101
Methyl 17(+)-hydroxystearate	2.07	95
3-Methyl-4-nonanol	0.84	92
2-Methyl-3-octanol	0.84	101
2-Methyl-3-pentanol	1.07	101
3-Methyl-1-pentanol	0.97	94
4-Methyl-4-penten-2-ol	2.00	97
5-Nonanol	2.00	100
4-Octanol	2.00	100
Oleyl alcohol	1.12	101
β-Phenylethanol	0.74	100

and nil in its absence. Analysis of the reaction mixture prior to addition of the base even after incubating for 24 hr showed practically no ester formation. Analysis of the reaction mixture within a minute after addition of the base showed that the reaction was complete upon addition of the base even for tertiary alcohols. The α-hydroxy acid methyl esters which do not react quantitatively could not be forced to react further by prolonged incubation of the complete reaction mixture.

Triethylenediamine was found to be much superior to pyridine as a catalyst for the reaction of alcohols with the reagent. Using cholesterol as the alcohol, pyridine gave only about a 20% yield of derivative under the same conditions that triethylenediamine gave a quantitative yield. Schenk *et al.* (16) also reported that triethylenediamine was much

superior to pyridine for base-catalyzed acetylation of hydroxyl groups.

The sequence of addition of the reagents can be varied only one other way while still maintaining quantitative acylation. Thus, the base and alcohol solutions can be dried together followed by addition of the acid chloride. In this sequence, as in the reverse sequence, a red color is produced followed by the solution becoming turbid and the gradual fading of the color to a lighter shade. The other possible sequence, i.e., mixing of acid chloride and base together followed by addition of the alcohol, gives no yield. We have preferred to use the sequence outlined under "Experimental" since alkaline-sensitive structures, especially esters, might be present in an unknown and give rise to artifacts.

Solvent. Although all of the derivatizations listed in Tables 1 and 2 were carried out in benzene, other solvents have been tried with success. Benzene and hexane, carbon tetrachloride and benzene, and methylene chloride and benzene combinations have given quantitative yields with many alcohols. Up to 1:1 mixtures of these solvents have been employed. It is, of course, necessary to purify the solvents although commercial carbon tetrachloride has been found to be completely free of compounds that react with pyruvic acid chloride 2,6-dinitrophenylhydrazone. Methylene chloride contains small amounts of reactive substances and also develops HCl on standing, which interferes with the reaction.

Interferences. Primary and secondary amines and thiols react with the reagent and interfere in the determination of alcohols. However, this situation can be circumvented by using the quantitative column procedure for separating the alcohol derivatives from amine and thiol derivatives (9). Methanol, however, will not be recovered with the other alcohol derivatives.

Water interferes by consuming reagent, but gives pyruvic acid 2,6-dinitrophenylhydrazone, which is strongly adsorbed on alumina and does not interfere with the determination of the esters. The effect of water can be overcome by drying in the manner described under "Experimental," or by increasing the ratio of acid chloride to alcohol. The appearance of the red color upon addition to the reaction mixture of triethylenediamine usually indicates that sufficient acid chloride is present to react with all of the alcohol and any water that is present in the reaction medium.

Organic acids interfere probably through the formation of mixed anhydrides with pyruvic acid chloride 2,6-dinitrophenylhydrazone. Figure 1 depicts the type and extent of inhibition of the reaction by lauric acid. Other fatty acids behave in an analogous manner under the same conditions. In very dilute solution (i.e., in 25 ml of benzene) the inhibitory effect of fatty acids is even more marked. The adverse effect of

organic acids on the reaction can be overcome by the addition of more acid chloride or, of course, by the removal of the acids prior to addition of the acid chloride. The interfering effect of organic acids on the acylation can be noted visually upon addition of triethylenediamine to the reaction mixture. When the concentration of organic acid is high enough to be inhibitory, addition of the base will cause no or only a fleeting red color instead of the usual relatively persistent deep red hue.

Carbonyl compounds and esters at the highest amounts tested do not interfere. Thus, 500 μmoles of nonanal and 600 μmoles of 2-nonanone did not inhibit. Ethyl heptanoate (580 μmoles) also caused no inhibition. It was necessary, however, to purify these three compounds prior to their addition to the reaction mixture since they originally contained sufficient organic acid impurities to inhibit the reaction.

Procedure for an unknown. When dealing with an unknown solution of alcohols, it is necessary to establish the concentration of acid chloride needed for quantitative acylation. This is readily accomplished by taking small, increasing volumes of the solution and adding a fixed amount of acid chloride to them essentially as was described for assaying known concentrations of alcohols. Plotting the amount of derivative obtained against volume of alcohol solution taken will give a straight-line relationship when the ratio of acid chloride to alcohol is satisfactory. The proper amount of acid chloride can then be added to the remainder of the solution followed by drying and the appropriate amount of triethylenediamine.

Limitations. The procedure as described is limited by the polarity of the alcohol and the derivative formed. Alcohols that are insoluble in benzene (or in methylene chloride) cannot be assayed as described. Derivatives which will not elute from the alumina with benzene can be eluted with more polar solvents such as chloroform or ethyl acetate and even alcohols without removing the excess reagents from the column. However, derivatives that are eluted with solvents more polar than benzene will be contaminated by an unidentified yellow substance which forms upon addition of the base to the acid chloride. Although the amount of this compound is small, it precludes the assay of very small amounts of alcohol forming a derivative which cannot be removed with a reasonable volume of benzene.

The acylation of polyhydroxy alcohols with the reagent has been examined superficially. Aside from solubility problems in the preferred system, the problem of quantitation appears to be one of obtaining complete acylation of vicinal hydroxyl groups. Other diols appear to be acylated to the bis derivative quantitatively. More work is anticipated on this problem.

SUMMARY

A colorimetric procedure is described for the quantitative determination of monohydric alcohols at the micromole level. The method is based on the acylation of the hydroxyl group with pyruvic acid chloride 2,6-dinitrophenylhydrazone in the presence of triethylenediamine and subsequent isolation of the derivative on alumina. The reaction is complete at room temperature upon addition of the base to the dried solution of the alcohol and acid chloride. Tertiary alcohols are quantitatively acylated as readily as primary and secondary alcohols. The procedure is applicable in both a small volume (1.8 ml) and a relatively large volume (25 ml) of benzene. Fatty acids inhibit the reaction.

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